Shedding patterns of verocytotoxin-producing Escherichia coli strains in a cohort of calves and their dams on a Scottish beef farm

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 2004, p. 7456–7465 Vol. 70, No. 12
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.12.7456–7465.2004

Shedding Patterns of Verocytotoxin-Producing *Escherichia coli* Strains in a Cohort of Calves and Their Dams on a Scottish Beef Farm

D. J. Shaw,[1]* C. Jenkins,[2,3]† M. C. Pearce,[1,4] T. Cheasty,[2] G. J. Gunn,[4] G. Dougan,[3] H. R. Smith,[2] M. E. J. Woolhouse,[1] and G. Frankel[3]

Veterinary Epidemiology Group, Centre for Tropical and Veterinary Medicine, College of Medicine and Veterinary Medicine, University of Edinburgh, Roslin, Midlothian,[1] Laboratory of Enteric Pathogens, Central Public Health Laboratory, Specialist and Reference Microbiology Division, Health Protection Agency, London,[2] Centre for Molecular Microbiology and Immunology, Imperial College, London,[3] and Epidemiology Unit, SAC Veterinary Science Division, Drummondhill, Inverness,[4] United Kingdom

Received 26 February 2004/Accepted 6 August 2004

Rectal fecal samples were taken once a week from 49 calves on the same farm. In addition, the dams of the calves were sampled at the time of calf birth and at the end of the study. Strains of verocytotoxin-producing *Escherichia coli* (VTEC) were isolated from these samples by using PCR and DNA probe hybridization tests and were characterized with respect to serotype, verocytotoxin gene (*vtx*) type, and the presence of the intimin (*eae*) and hemolysin (*ehxA*) genes. A total of 170 VTEC strains were isolated during 21 weeks from 130 (20%) of 664 samples from calves and from 40 (47%) of 86 samples from their dams. The characteristics of the calf strains differed from those strains isolated from the dams with respect to verocytotoxin 2 and the presence of the *eae* gene. In addition, no calf shed the same VTEC serogroup (excluding O?) as its dam at birth or at the end of the study. The most frequently detected serogroups in calves were serogroup O26 and provisional serogroup E40874 (VTEC O26 was found in 25 calves), whereas in dams serogroup O91 and provisional serogroup E54071 were the most common serogroups. VTEC O26 shedding appeared to be associated with very young calves and declined as the calves aged, whereas VTEC O2 shedding was associated with housing of the animals. VTEC O26 strains from calves were characterized by the presence of the vtx_1, *eae*, and *ehxA* genes, whereas vtx_2 was associated with VTEC O2 and provisional serogroup E40874. The high prevalence of VTEC O26 and of VTEC strains harboring the *eae* gene in this calf cohort is notable because of the association of the O26 serogroup and the presence of the *eae* gene with human disease. No association between calf diarrhea and any of the VTEC serogroups was identified.

Verocytotoxin (VT)-producing *Escherichia coli* (VTEC) causes a wide spectrum of diseases in humans ranging from mild diarrhea to severe diseases, such as hemorrhagic colitis and hemolytic-uremic syndrome (17). The production of VT by *E. coli* was first described by Konowalchuk et al. (20). Subsequent studies described two types of VT, VT1 and VT2, and showed that the VT gene (*vtx*) is phage encoded (27, 39). VTEC is also referred to as Shiga toxin-producing *E. coli*. There are seven established *vtx* subtypes, including vtx_1 (subtype), vtx_{1c} (53), vtx_2 (subtype), vtx_{2c}, vtx_{2d}, vtx_{2e}, and vtx_{2f} (37).

Strains of VTEC belonging to serotype O157:H7 and nonmotile serotype O157 (O157:H-) are most commonly associated with severe human disease in the United Kingdom (47). However, infections with other VTEC strains, including strains belonging to serogroups O26, O103, O111, and O145, have been detected (48), and outbreaks caused by VTEC serogroups other than O157 have been described outside the United Kingdom (4, 24, 31). Some VTEC strains have the ability to cause attaching and effacing lesions in the human intestine and carry the intimin or *E. coli* attaching and effacing (*eae*) gene (16), which is part of a pathogenicity island called the locus of enterocyte effacement and is responsible for intimate attachment of VTEC to the intestinal epithelial cells (10, 16). There are at least 10 different intimin subtypes, including alpha (α), beta (β), gamma (γ), delta (δ), epsilon (ε), iota (ι), kappa (κ), theta (θ), zeta (ζ), and eta (η) (54). Some VTEC strains also produce a plasmid-encoded VTEC hemolysin (*ehxA*) (38). Strains of VTEC carrying the vtx_2, vtx_{2c}, and *eae* genes are thought to be associated with more severe human disease (11, 33).

VTEC, including serogroup O157, can be isolated from the feces of cattle, which represent an important source of human infection (1, 19). There is no evidence that VTEC is pathogenic for adult cattle, although some serogroups can cause diarrhea in calves (9, 36, 40, 41). Humans can become infected with VTEC by ingesting contaminated food or water or by transmission from infected animals or humans (47). However, it is not yet clear if all VTEC strains found in cattle and other animals are pathogenic for humans (31).

Previous studies have shown that VTEC strains harboring the vtx_1 and *eae* genes are isolated more frequently from calves than from adult cattle (2, 46). The epidemiology of VTEC in calves is interesting because of the possible association between certain VTEC strains and diarrhea in young animals (9, 36, 40, 41) and because *eae*-positive VTEC strains are isolated more commonly from patients with hemolytic-uremic syn-

* Corresponding author. Mailing address: Faculty of Veterinary Medicine, College of Medicine and Veterinary Medicine, University of Edinburgh, Roslin, Midlothian EH25 9RG, United Kingdom. Phone: 44 (0) 131 6507573. Fax: 44 (0) 131 651 3903. E-mail: Darren.Shaw@ed.ac.uk.

† Present address: Microbiology, Royal Free Hospital, London NW3 2QG, United Kingdom.

drome than from patients with less severe diseases (3, 52). Furthermore, over the first couple of months of calf life the gut physiology changes and the immune system develops (13, 35), yet very little is known about how this alters the presence and characteristics of VTEC in calves.

A large study was carried out on a Scottish farm, on which rectal fecal samples were taken beginning at birth once a week from 49 calves on the same farm over a 5-month period. An analysis of the epidemiology on this farm of *E. coli* serogroups O26, O103, and O145, as detected by immunomagnetic separation (IMS), has been described previously (32). Further investigations in which PCR and DNA probes were used to detect *vtx* genes showed that during this study, the calf cohort shed many other VTEC serogroups. In this paper we describe the isolation of all strains of VTEC by a PCR-DNA probe hybridization method (which detects *vtx* genes) and the shedding patterns of VTEC serogroups in the herd.

MATERIALS AND METHODS

Fecal sampling. Per rectum fecal samples for this study were taken from 49 calves and their dams ($n = 44$) on a beef cattle farm in northern Scotland. The calves were born between August and November 2001 and were sampled weekly from birth until the end of the study in January 2002, except for a fortnight around Christmas (weeks 18 and 19) (Fig. 1). One calf died shortly after birth and was sampled once; a second calf died 4 weeks after birth and was sampled three times. Most dams were sampled at the time of calving and at the end of the sampling period; the exceptions were one dam which was sampled only at calving, one dam which was sampled only at the end of the sampling period, and a third dam which was sampled neither at calving nor at the end of the sampling period (Fig. 1). A total of 664 samples were taken from calves, and 86 samples were taken from dams. The presence or absence of calf diarrhea was noted in calves at the time of sampling. Sample collection was carried out in accordance with United Kingdom Government Home Office license requirements. From August until 9 November, the study animals were kept together in the same field on pasture. After 9 November the group was housed in a barn on straw bedding.

Identification of samples containing verocytotoxin genes by PCR. Within 48 h of sampling, 1 g of feces from each sample was suspended in 4 ml of phosphate-buffered saline, and 200 μl was added to 10 ml of buffered peptone water and incubated for 18 h at 37°C. The cultures were plated on MacConkey agar and incubated for 18 h at 37°C. Nutrient broths were inoculated with a sweep of mixed colonies from the MacConkey agar plates, incubated at 37°C for 2 to 4 h, and examined for the presence of the vtx_1 and vtx_2 genes by PCR, as described previously (47).

Isolation of VTEC by DNA hybridization. Mixed colonies from an original MacConkey agar plate, which were shown to contain verocytotoxin genes by PCR, were transferred by replica plating onto a nylon membrane placed on a nutrient agar plate prior to incubation at 37°C for 4 to 6 h. The membrane was prepared for hybridization by the method of Maniatis et al. (23). Individual VTEC colonies were identified by colony DNA hybridization with a mixture of vtx_1 and vtx_2 polynucleotide probes (43, 50). Up to five VTEC colonies detected by the probe were marked on the master plate and inoculated onto MacConkey agar. PCR was carried out as described above with pure growth to confirm that VTEC had been isolated.

Serotyping. Each isolate was serotyped by using the scheme described by Kauffman (18), which depends on identification of the heat-stable lipopolysaccharide somatic (O) and flagellar (H) antigens (14).

Strain characterization. Subtyping of *vtx* was carried out by using digoxigenin-labeled DNA probes for vtx_1 and vtx_2 (50). *vtx* subtyping was performed by using the PCR-restriction fragment length polymorphism method described by Lin et al. (21). The *eae* gene and the *ehxA* gene were detected by using PCR (30, 38), and intimin subtypes were determined by using a PCR-restriction fragment length polymorphism method described by Jenkins et al. (15).

Statistical analysis. All analyses were carried out by using S-plus (Insightful Corp, Seattle, Wash.). When we considered the changes with age or time with respect to (i) VTEC-positive samples or (ii) virulence factors and serogroups excreted for VTEC-shedding calves, generalized linear mixed effect models with binomial errors (to account for the binary data) were used, with a logit link between the observed binomial and unobserved normal scales (34). The calf identification number was entered as a random factor (to account for nonindependence of samples). Age and time were then added as linear covariate fixed effects, and whether calves were housed was added as a two-level class fixed effect. For the majority of analyses fixed effects were first added to univariate models singularly. When no fixed effects were significant, then only univariate results were reported. If more than one fixed effect was significant at the 5% level, the effects were added in a multivariate model in order of greatest significance, and the relative contributions of the fixed effects and their interactions were evaluated. In such cases the multivariate results are also reported below.

We were also interested in ascertaining the significance of any association among the presence of virulence factors, serogroups, and whether a sample came from a calf or a dam. Ideally, just the shedding by calves at birth and at the end of the study compared to the shedding by their dams at the same time would have been compared. However, there were very few calves that shed VTEC at these times. Therefore, generalized linear mixed-effect models with binomial errors were used to look at the presence of VTEC or virulence factors in calves and dams. The cattle identification number was entered as a random variable to account for the nonindependence of samples. Odds ratios (OR) (with 95% confidence limits [95% CI] [8]) were calculated to determine whether a sample that came from a dam was more or less likely to have particular virulence factors than the samples from its calves.

Generalized linear models with Poisson errors were used to look at changes in the number of different VTEC serogroups excreted in a particular week (either sampling or age) or to see whether more serogroups were excreted during the housed period than during the nonhoused period (7). Data were square root transformed prior to analysis to obtain normalized residuals. Different numbers of samples were examined for a given week or age class, so the analysis was weighted by the number of samples examined per week. In addition, there was nonindependence of samples when we looked at the number of different serogroups observed in a week. Therefore, a dummy variable of the number of serogroups observed in the previous week was created as described by Synge et al. (42). This information was added to the statistical model prior to age or sampling time. In all cases a P value of <0.05 was taken to indicate significance.

RESULTS

Summary of strains isolated. A total of 170 VTEC strains were isolated during 21 weeks from 47% of the dam samples (40 of 86 samples) and 20% of the calf samples (130 of 664 samples) (Tables 1 and 2 and Fig. 1). The proportion of VTEC-positive samples isolated from calves was significantly lower than the proportion of VTEC-positive samples isolated dams ($P < 0.001$, regression coefficient [rc] = -0.649), and the OR indicated that samples from dams were 3.63 (95% confidence limit, 2.27 to 5.80) times more likely to be VTEC positive than samples from calves.

The most commonly isolated VTEC serogroup obtained from dams was serogroup E54071 (Table 1 and Fig. 1) (the prefix E indicates a provisional new serogroup that has not yet been designated a formal O group), whereas the most frequently detected VTEC serogroup in calves was serogroup O26 (Table 2). Sixteen VTEC strains from dams and 35 VTEC strains from calves that could not be typed by the current serotyping scheme were designated O?. VTEC serogroup O157 was not detected by the PCR-DNA probe method on this farm during this study. Of the five typeable serogroups that were detected in the dams, four were also detected in the calves (O26, O91, O113, and E54071). However, no calf excreted the same VTEC (excluding O?) as its dam at the time of calf birth (Fig. 1). In addition, 9 of 13 typeable VTEC serogroups obtained from the calves were not detected in any of the dams.

Shedding patterns in calves. During the course of the study there was no age-related change in the percentage of calves excreting VTEC ($P = 0.514$, $rc = 0.002$), and the prevalence was around 25% (Fig. 2a). While the percentage of calves

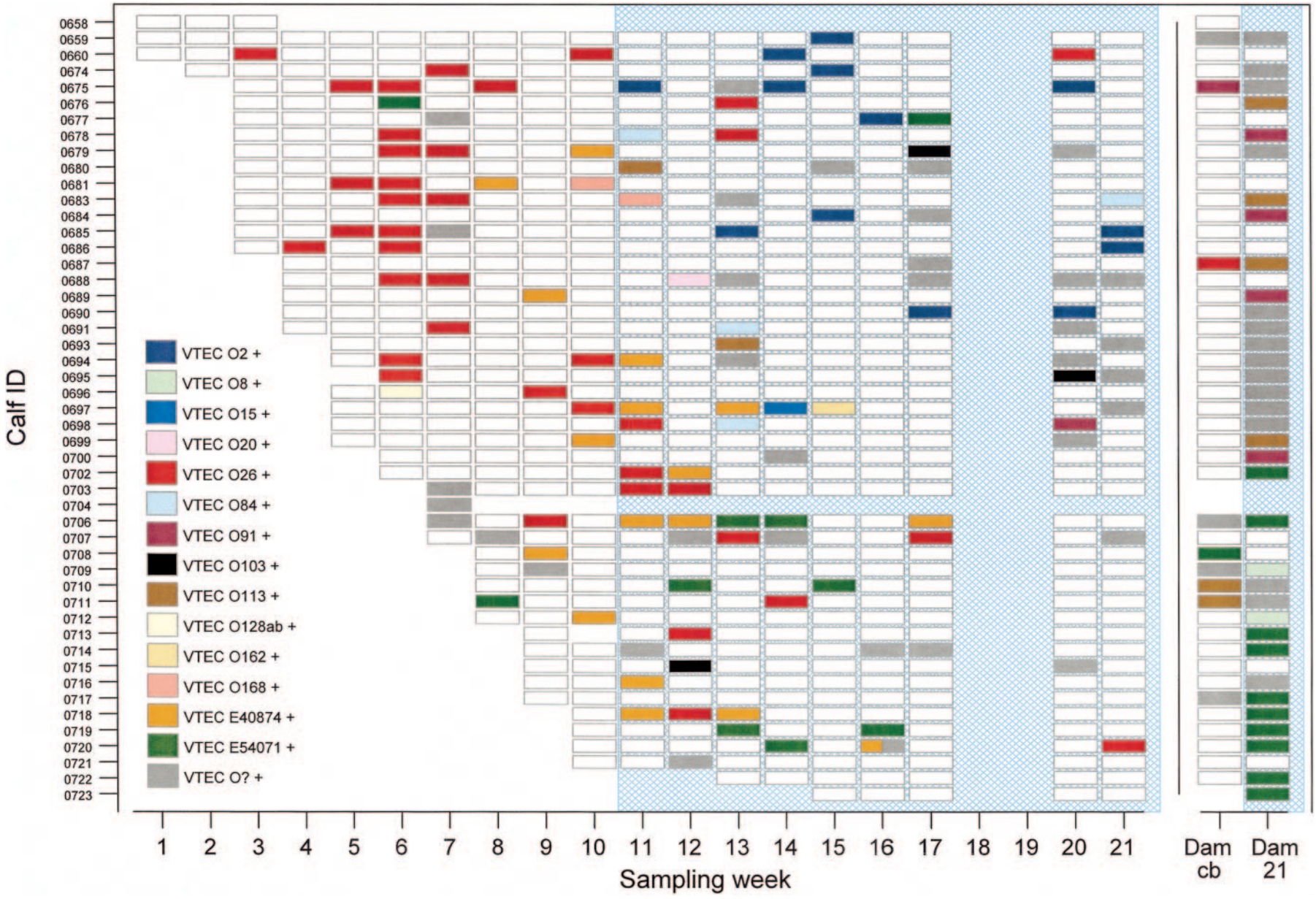

FIG. 1. Summary, by sampling week, of VTEC serogroups isolated from the 49 calves during the course of the study. Also included are the VTEC serogroups detected in the dams at the time of birth of the calves (Dam cb) and at the end of the study (Dam 21). Open boxes indicate weeks when samples were taken but no isolates were obtained. No sampling occurred during weeks 18 and 19. The cross-hatched areas show when all cattle on the farm were housed (9 November onward). Calf ID, calf identification number.

TABLE 1. Characteristics of verocytotoxin-producing *E. coli* isolated from dams on a Scotland beef farm between August 2001 and January 2002[a]

Serogroup[b]	Week isolated	*vtx* type	vtx_2 subtype	*eae* (subtype)	*ehxA*
O8 (2)[c]	21	2	2 + 2c	−	+
O26 (1)	3	1		+ (β)	+
O91 (1)	3	2	2 + 2c	−	+
O91 (4)	21	2	2 + 2c	−	+
O113 (1)	8	2	2c	−	−
O113 (2)	21	2	U[d]	−	−
O113 (2)	21	2	2	−	+
E54071 (1)	8	1 + 2	2	−	+
E54071 (10)	21	1 + 2	2	−	+
O? (1)	1	2	2e	−	+
O? (1)	8	2	2 + 2c	−	+
O? (1)	10	2	2 + 2c	−	+
O? (11)	21	2	2 (2), 2c (2), 2 + 2c (7)	−	+
O? (1)	7	1 + 2	2	−	−
O? (1)	21	2	2 + 2c	−	−

[a] Samples were collected either at the time of calf birth (weeks 1 to 8) or at the end of the study (week 21).
[b] The prefix E indicates a provisional new serotype that has not yet been designated a formal O group. Strains that could not be typed by the current serotyping scheme were designated O?.
[c] The numbers in parentheses are the numbers of strains isolated.
[d] U, PCR-restriction fragment length polymorphism pattern does not conform to any known *vtx* subtype.

excreting VTEC slowly increased to around 20% by January 2002 (Fig. 2b), the increase over time was not significant ($P = 0.732$, $rc = <0.001$). In addition, being housed did not make a difference in the percentage of calves excreting VTEC strains ($P = 0.302$, $rc = 0.105$). Significantly fewer serogroups were isolated as calves aged (Fig. 2c) ($P = 0.037$, $rc = -0.024$), but multivariate analyses revealed that there was a significant interaction between housing status and age ($P < 0.001$, $rc = -0.099$); there was no change with age during the nonhoused period ($P = 0.684$, $rc = 0.009$), but there was a significant decrease with age when calves were housed ($P < 0.001$, $rc = -0.191$). While there was no significant change in the number of serogroups (excluding O?) with sampling time (Fig. 2d) ($P = 0.289$, $rc = 0.015$), calves excreted significantly more serogroups during the housed period ($P = 0.032$, $rc = 0.833$). Furthermore, multivariate results revealed that there was an association between housing status and time of sampling ($P = 0.004$, $rc = -0.066$); there was a significant increase in the number of serogroups excreted during the nonhoused period ($P = 0.008$, $rc = 0.147$), and there was a nonsignificant decrease during the housed period ($P = 0.101$, $rc = -0.026$).

TABLE 2. Characteristics of verocytotoxin-producing *E. coli* strains isolated from a cohort of calves on a Scotland beef farm between August 2001 and January 2002

Serogroup[a]	*vtx* type	vtx_2 subtype	*eae*	*ehxA*
O2 (10)[b]	2	2 (8), 2c (2)	−	+
O2 (2)	2	2	−	−
O15 (1)	2	2	−	+
O20 (1)	2	2c	−	−
O26 (35)	1		+ (β)	+
O26 (1)	2	U[c]	+ (β)	+
O26 (4)	1 + 2	2 (3), 2c (1)	+ (β)	+
O84 (4)	1		+ (ζ)	+
O91 (1)	2	2 + 2c	−	+
O103 (1)	2	2	+ (θ)	+
O103 (1)	2	2	−	+
O103 (1)	2	2	−	−
O113 (2)	2	2c (1), U (1)	−	−
O128ab (1)	1		−	−
O162 (1)	2	2 + 2c	−	−
O168 (2)	2	2 + 2c	−	−
E40874 (17)	2	2c (16), 2 + 2c (1)	+ (β)	+
E54071 (10)	1 + 2	2	−	+
O? (2)	2	2c	+ (β)	+
O? (11)	2	2 (2), 2 + 2c (11)	−	+
O? (21)	2	2 (10), 2 + 2c (10), U (1)	−	−
O? (1)	1 + 2	2	−	−

[a] The prefix E indicates a provisional new serotype that has not yet been designated a formal O group. Strains that could not be typed by the current serotyping scheme were designated O?.
[b] The numbers in parentheses are the numbers of strains isolated.
[c] U, PCR-restriction fragment length polymorphism pattern does not conform to any known *vtx* subtype.

Altogether, VTEC strains were detected in 45 of the 49 calves by the PCR-DNA hybridization technique (Fig. 1). Four main serogroups were detected, O26 (24 isolates), O2 (8 isolates), E40874 (13 isolates), and E54071 (7 isolates), but other serogroups also were detected in more than one calf, including O84 (4 isolates), O103 (3 isolates), O113 (2 isolates), and O168 (2 isolates) (Fig. 1). VTEC O26 was the serogroup that was most frequently isolated from calves born during the first 7 weeks of the study, whereas in the calves born between week 7 and week 10, serogroups E40874 and E54071 were dominant. Shedding of VTEC O26 decreased both as the age of the calves increased ($P < 0.001$, $rc = -0.029$) (Fig. 3a) and with time of sampling ($P < 0.001$, $rc = -0.043$) (Fig. 3b). There was also a significant difference in VTEC O26 shedding between nonhoused and housed calves ($P = 0.005$, $rc = 1.081$) (Fig. 3b), and most of the O26 was shed prior to housing. Multivariate results revealed no interaction between time and age ($P < 0.001$), and housing status did not alter the age-related decline in O26 shedding ($P = 0.146$, $rc = 0.018$). There was an overall increase in the number of calves shedding VTEC O2 with age ($P < 0.001$, $rc = 0.056$) (Fig. 3c) and time ($P < 0.001$, $rc = 0.052$) (Fig. 3d). However, no O2 strains were detected before the calves were housed (Fig. 3d), and if only the data for the housed period were included, then O2 shedding was not age dependent ($P = 0.499$, $rc = 0.008$) or time dependent ($P = 0.521$, $rc = -0.008$). There was also an apparent decline in VTEC E40874 shedding with increasing calf age ($P = 0.039$, $rc = -0.017$) (Fig. 3e). No effect of housing status was observed ($P = 0.956$, $rc = -0.014$). In addition, no linear time-dependent relationship was observed ($P = 0.192$, $rc = -0.010$) (Fig. 3f); however, 88% of the E40874 shedding occurred in the middle of the study. Therefore, if a quadratic function of time was fitted first to capture this shedding pattern ($P < 0.001$, $rc = -0.001$), then E40874 shedding was not age dependent ($P = 0.148$, $rc = -0.032$). The shedding pattern of VTEC E54071 was not significantly associated with age, time, or housing status ($P > 0.270$, $rc < -0.012$) (Fig. 3g and h).

Presence of *vtx* genes. The vtx_1 genes were detected in 13 of 40 (33%) strains from dams and in 55 of 130 (42%) calf strains, and there was no significant difference between calves and dams ($P = 0.240$, $rc = -0.226$, OR = 0.64 [OR 95% CI, 0.30 to 1.35]). In contrast, vtx_2 genes were found in 39 (98%) and 90 (69%) of the strains from dams and calves, respectively, and

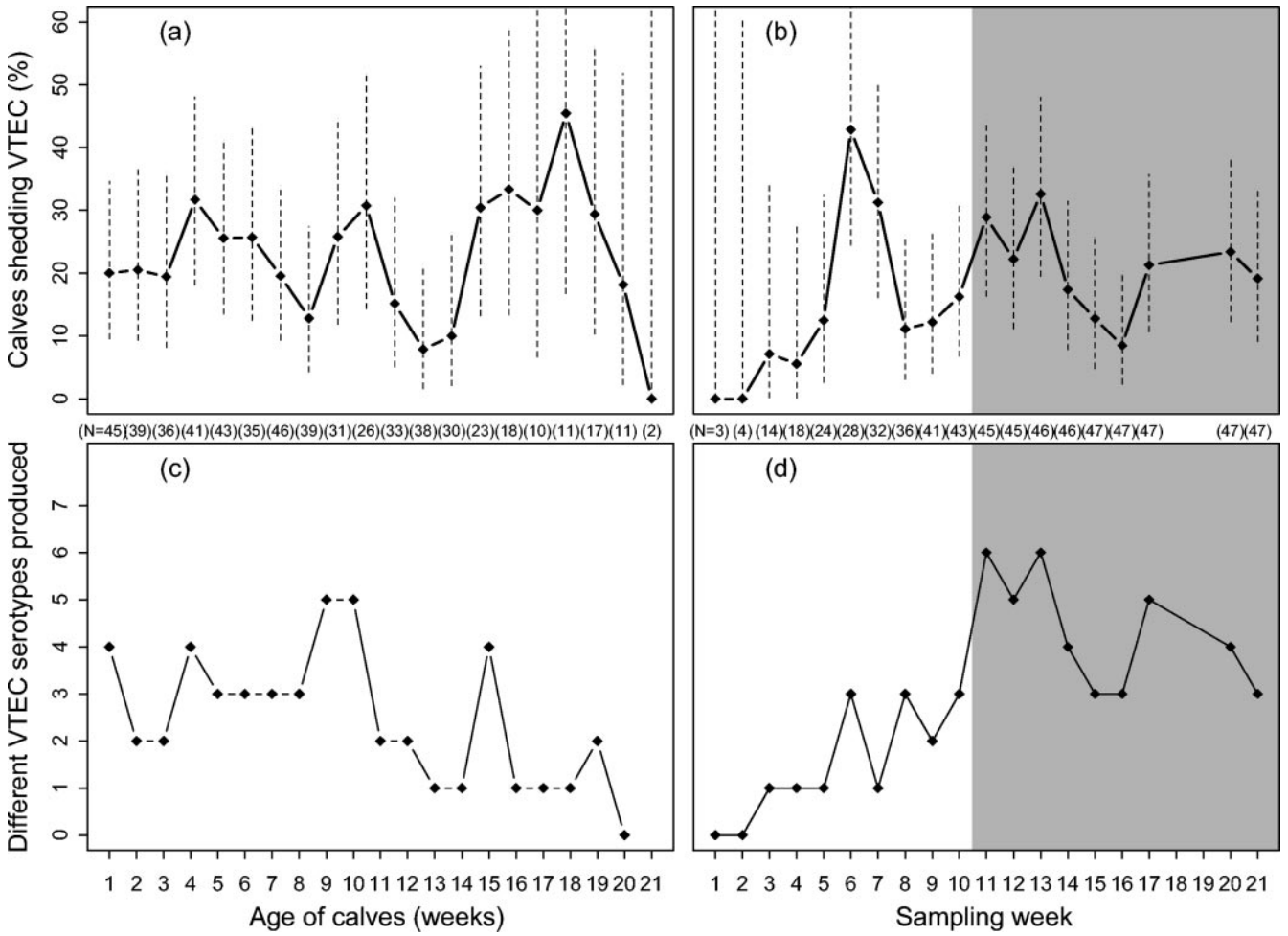

FIG. 2. (a and b) Changes in the percentage of calves excreting VTEC by calf age (a) and sampling week (b). The vertical dashed lines indicate 95% confidence intervals for the percentages, and the numbers in parentheses are the numbers of calves that were sampled in the different weeks. (c and d) Changes in the number of different typeable VTEC serogroups (excluding O?) excreted as a function of calf age (c) and sampling week (d). The shaded areas in panels b and d indicate when all cattle on the farm were housed (9 November onward).

VTEC strains from dams were significantly more likely to harbor vtx_2 genes than VTEC strains from calves were ($P = 0.006$, $rc = 1.444$, OR = 17.97 [OR 95% CI, 2.35 to 137.3]).

VTEC O26 strains from calves were characterized by the presence of vtx_1, *eae*, and *ehxA* genes ($P < 0.001$), whereas vtx_2 was associated with VTEC O2 and provisional serogroups E40874 and E54071 ($P < 0.032$). There was a significant decline in the percentage of VTEC strains from calves harboring the vtx_1 gene with age ($P < 0.010$, $rc = -0.026$) (Fig. 4a), from a peak when calves were between 3 and 6 weeks old to almost zero (except for two 19-week-old calves) in calves older than 12 weeks. There were also fewer VTEC strains harboring vtx_1 genes over the sampling time ($P < 0.001$, $rc = -0.031$) (Fig. 4b) and during the housed period ($P < 0.002$) (Fig. 4b). The multivariate results revealed that there was no interaction between the rate of decline with age and housing status ($P = 0.293$, $rc = 0.012$) or between age and sampling time ($P = 0.625$, $rc < 0.001$). In animals older than 7 weeks, vtx_2 was the dominant gene type, and there was a significant increase in the percentage of VTEC strains harboring vtx_2 genes with calf age ($P = 0.002$) (Fig. 4c); however, once the higher percentage of VTEC strains harboring vtx_2 genes during the housed period ($P < 0.001$) (Fig. 4d) was taken in to account, there was no significant change in vtx_2 detection and calf age ($P = 0.139$). If only VTEC strains harboring vtx_2 gene subtype vtx_2 were considered, then again there was an increase with calf age ($P = 0.003$), but this increase with age was significantly steeper during the housed period ($P = 0.002$). No age-, time-, or housing status-related patterns were observed for vtx_{2c} ($P > 0.052$).

Presence of *eae* and *ehxA* genes. The *eae* genes were detected in one strain (serogroup O26) from dams and in 64 (50%) of the strains (serogroups O26, O84, and O103 and provisional serogroup E40874) from calves; i.e., VTEC strains carrying the *eae* genes were significantly associated with calves ($P < 0.001$, $rc = -1.801$, OR = 0.03 [OR 95% CI, 0.01 to 0.21]). There was no significant association between dams and the presence of *ehxA* genes ($P = 0.080$, $rc = 0.453$, OR = 2.53 [OR 95% CI, 0.91 to 7.04]) (Tables 1 and 2).

The percentage of VTEC strains harboring *eae* significantly declined with calf age ($P = 0.008$, $rc = -0.033$) (Fig. 4e) and time ($P < 0.001$, $rc = -0.038$) (Fig. 4f). However, significantly fewer VTEC strains harboring *eae* were detected during the

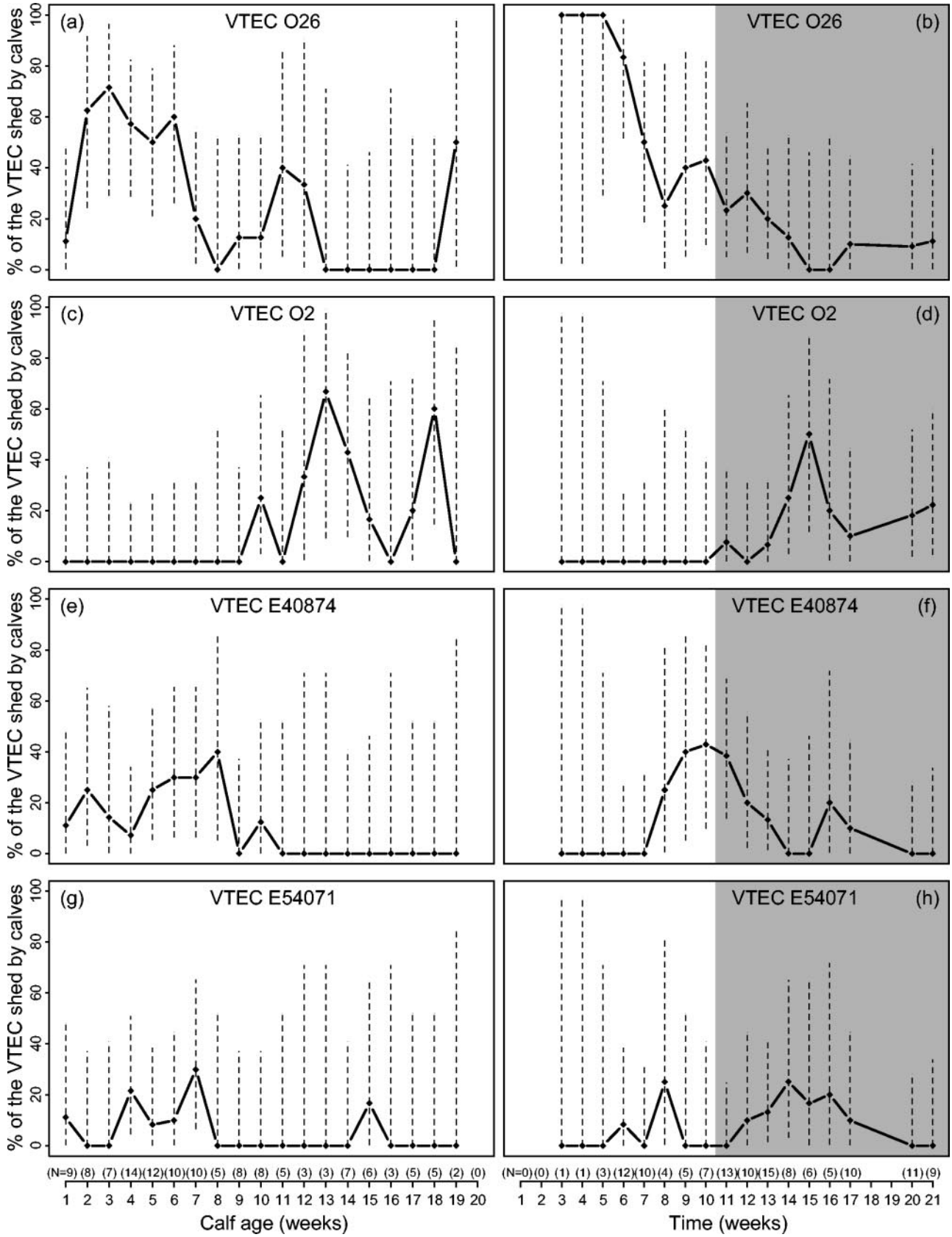

FIG. 3. Changes in the percentage of VTEC-shedding calves that shed O26 (a and b), O2 (c and d), E40874 (e and f), and E54071 (g and h) by calf age (a, c, e, and g) and sampling time (b, d, f, and h). The vertical dashed lines indicate 95% confidence intervals for the percentages, and the numbers in parentheses are the numbers of VTEC strains (of a total of 130 strains) that were detected in calves that were sampled for each week.

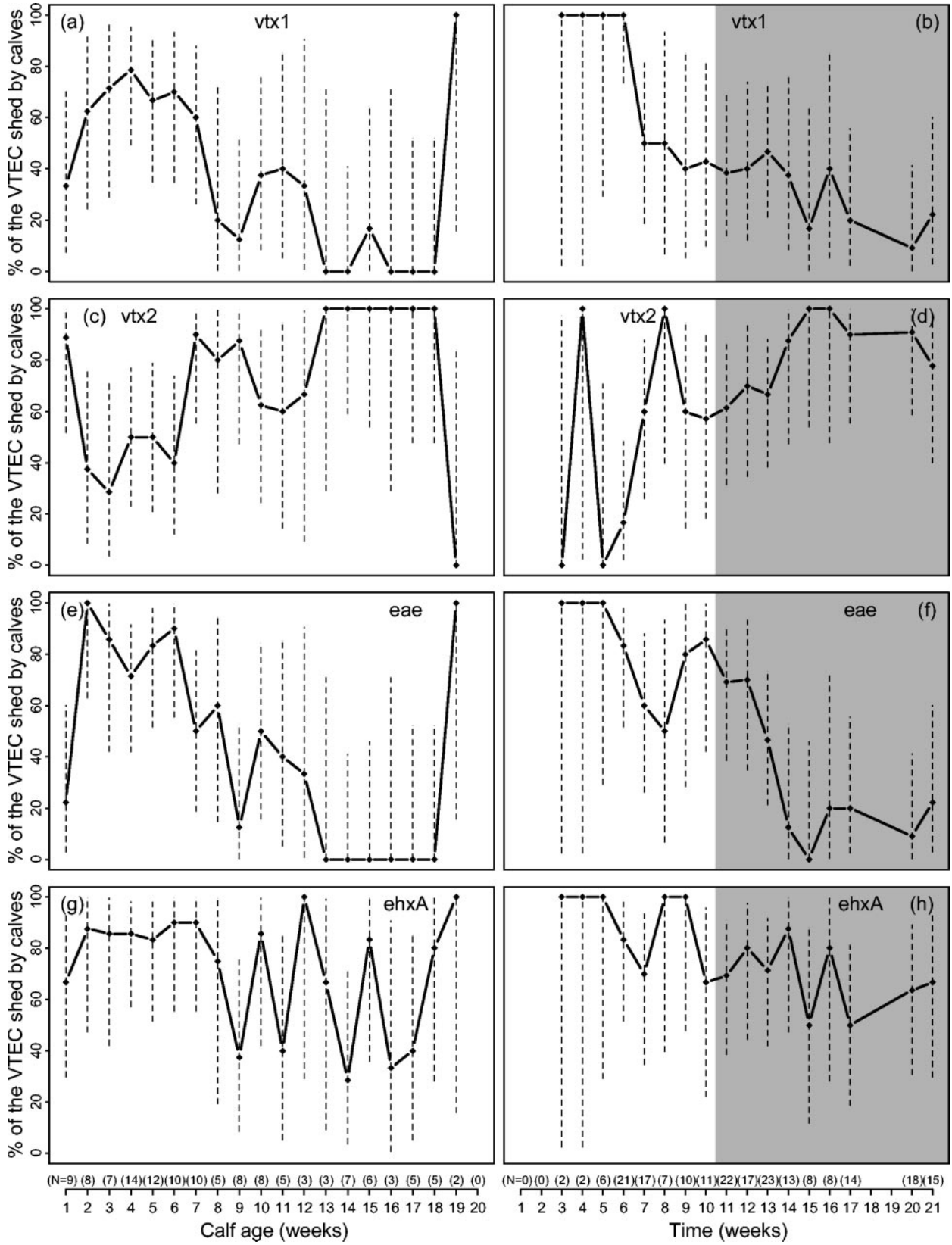

FIG. 4. Changes in the percentage of VTEC-shedding calves that shed vtx_1 (a and b), vtx_2 (c and d), *eae* (e and f), and *ehxA* (g and h) by calf age (a, c, e, and g) and sampling time (b, d, f, and h). The vertical dashed lines indicate 95% confidence intervals for the percentages, and the numbers in parentheses are the numbers of VTEC strains (of a total of 130 strains) that were detected in calves that were sampled for each week. Shaded areas are as for Fig. 2.

housed period ($P = 0.012$, $rc = -1.83$) (Fig. 4f), and multivariate analyses revealed an interaction between housing status and age of the calf, with a steeper age-related decline during the housed period ($P = 0.007$, $rc = -0.115$). Sampling time was no longer significant if housing status was considered first ($P = 0.510$, $rc = -0.021$). The percentage of VTEC strains harboring *ehxA* genes significantly declined with calf age ($P < 0.034$, $rc = -0.012$) (Fig. 4g) and with sampling time ($P = 0.048$, $rc = -0.012$) (Fig. 4h) but not with housing status ($P = 0.092$) (Fig. 4h). There was no interaction between calf age and sampling time ($P = 0.552$, $rc < 0.001$). The most common intimin subtype was β-intimin (serogroup O26 and provisional serogroup E40874), which was present in 92% of the *eae*-positive calf strains. There were also four strains (all serogroup O84) which carried the ζ-intimin gene, and one O103 strain carrying θ-intimin was detected (Table 2).

In dams, there were no significant associations between the detection of vtx_1 and the detection of either *eae* or *ehxA* ($P > 0.325$) or between the detection of vtx_2 and the detection of *ehxA*. However, there was a negative association between the detection of vtx_2 and the detection of *eae* ($P = 0.025$), and no vtx_2^+ strains were eae^+. The detection of vtx_1 was positively associated with the detection of *eae* and *ehxA* in VTEC strains from calves ($P < 0.001$), whereas the detection of vtx_2 was negatively associated with the detection of *eae* and *ehxA* ($P < 0.003$). In addition, the detection of *ehxA* was positively associated with the detection of the *eae* gene in the VTEC strains ($P < 0.001$).

Calf diarrhea. Throughout the study, 14 calves showed evidence of diarrhea. However, VTEC (serogroup O26) was detected in only one calf at the same time as diarrhea. One week before diarrhea was observed, two calves had VTEC in their fecal samples (one had serogroup O26, and the other had provisional serogroup E54071).

DISCUSSION

The results presented here are results for one cohort of calves and dams on a single farm and should therefore be interpreted with some caution. Furthermore, the calves were born throughout the first half of the study, and all animals were housed in the second half of the study, leading to possible confounding among time of sampling, age of the calf, and housing status. Nevertheless, such studies are rare, and interesting patterns were observed. While samples were collected from dams only at the time of calf birth and at the end of the study, the proportion of samples containing VTEC was higher for dams (46%) than for calves (20%); this is in contrast to the results of other studies, in which the incidence of VTEC in calves was usually higher than the incidence of VTEC in adult cattle (2, 5, 45, 51). Cobbold and Desmarchelier (5) suggested that dams shed more VTEC during and after calving, because pregnancy and calving would be expected to be a stressor for the cows. However, they did not find any obvious correlation between calving and the number of animals shedding VTEC (5).

Thirteen different, typeable VTEC serogroups were detected in the calves (Fig. 1), whereas only five typeable VTEC serogroups were detected in the dams sampled at the time of calf birth and at the end of the study. The most frequently isolated VTEC serogroup in calves was VTEC O26. In the study of Pearce et al. (32) with the same cohort, in which IMS rather than PCR was used to detect *E. coli* serogroups O26, O103, O111, O145, and O157, VTEC O26 was also the most frequently isolated serogroup. However, IMS was shown to be 2.5 times more sensitive (for this serogroup) than PCR. Calves were kept together (either in the same area of pasture or, once housed, in the same area of the shed), which could have facilitated the spread of strains of VTEC from calf to calf. However, it is interesting that other serogroups did not appear to be distributed in the calf population in the same way as VTEC O26. This serogroup has been isolated from healthy adult cattle and calves in many studies (1, 2, 5, 32), although it has also been associated with diarrheal disease in calves (9, 40, 41). In a study of diarrheic calves, Orden et al. (28) found that the most common VTEC serogroup in calves was VTEC O26 (30%). Cobbold and Desmarchelier (6) looked for VTEC on three farms in Australia and found that 7.8% of the isolates were VTEC O26:H11.

The apparent association of VTEC O26 with calves that were between 1 and 7 weeks old in this study may simply reflect the serogroups present in the environment at the time of birth, although calves born when other serogroups predominated also shed VTEC O26. While this finding may be specific to this cohort, the replacement of one serogroup with another as the calves' ages increased may have been due to changes in the calf gut and the availability of niches for particular VTEC populations. For example, Naylor et al. (26) found recently that VTEC O157 preferentially occupied a niche at the recto-anal junction of the calf gut in calves that were 8 to 14 weeks old.

It has been suggested that VTEC O26 and VTEC harboring vtx_1 and *eae* are associated with diarrheagenic calves (9, 22, 25, 29, 46). The serotypes reported to be associated with diarrhea in calves include O5:H-, O8:H8, O20:H19, O26:H11, O103:H2, O111:H-, O118:H16, O128:H-, and O145:H- (22, 28). In this study, there was no association between calf diarrhea and any of the VTEC serotypes or their characteristics.

The analysis of the associations between serogroups and virulence factors also revealed that there appeared to be a number of distinct serogroup-virulence factor combinations that were observed in many calves in the calf cohort at certain times in the study. The decline in strains harboring vtx_1 genes with calf age and the increase in vtx_2-positive strains were related to the decline in VTEC O26 strains, which in this study and characteristically have the vtx_1 genes, whereas the increase in VTEC O2 strains later in the study was associated with an increase in detection of the vtx_2 genes. There was also a population of VTEC E40874 strains which were vtx_2^+, $ehxA^+$, and eae^+ that was present in young calves and then declined. Finally, a population of VTEC E54071 strains (vtx_1^+ vtx_2^+ $ehxA^+$) was detected in older calves.

By using the methods described in this study, all VTEC serogroups could be detected if bacteria representing the serogroups were present in sufficient numbers, and therefore, fecal samples containing more than one VTEC serogroup could be identified. Despite this, different VTEC serogroups were isolated from only one sample (Fig. 1). Pearce et al. (32) also found that concurrent shedding of more than one of the five serogroups considered was uncommon. These data and the present data suggest that cattle shed a single predominant

VTEC serogroup (and associated virulence factors) at any one point.

Previous studies have shown that calves as young as 48 to 72 h old excrete VTEC (5) and that a calf is more than twice as likely to shed VTEC if it is born to a VTEC-positive cow, although a VTEC-negative cow has an equal risk of producing either a VTEC-positive calf or a VTEC-negative calf (12). This suggests that calves can acquire VTEC from sources other than their dams (5, 32). In our study, the same VTEC serogroup was isolated from the calf and its dam on only four occasions, but the serogroups were all O? and in three of the four cases the concurrent shedding occurred at the end of the study (Fig. 1). VTEC serogroups O26, O91, O113, and E54071 detected in fecal samples from dams at the time of calf birth were found in other calves, perhaps indicating that calves may acquire strains of VTEC from adult cattle other than their dams. In this cohort, some VTEC serogroups were detected in calves but not isolated from any of the dams, suggesting VTEC acquisition from other sources. Alternatively, serogroups that are dominant in adult cattle may be different from the dominant serogroups in calves (e.g., O26 and E40874). The DNA hybridization method can detect between 0.1 and 760 organisms per g of feces (49), but much larger amounts of feces per sample and more sensitive tests would have to be employed to exclude dams as the source of calf infection.

This study provided data on the characteristics of strains of VTEC isolated from calves and the changes in shedding patterns of certain VTEC serogroups in the cohort. While far fewer samples were collected from dams, the characteristics of the calf strains differed from the characteristics of strains isolated from their dams with respect to serogroup, VT type, and the presence of the *eae* gene. Furthermore, the relative occurrence of VTEC serogroups and virulence factors changed as calves aged, with vtx_1-positive strains replaced by vtx_2-positive strains, and depended on whether calves were housed or on pasture. The most frequently detected VTEC serogroup was O26, a serogroup also associated with human disease (24, 44, 47). Characteristics of this serogroup, such as possession of the β-intimin gene, may influence its ability to colonize young cattle. Our results also suggest that the environment may have an influence on the shedding of VTEC in calves. Further cohort studies are required to ascertain whether the results obtained here are cohort specific or reflect a general pattern of VTEC epidemiology in calves.

ACKNOWLEDGMENTS

This study is part of an International Partnership Research Award in Veterinary Epidemiology (IPRAVE) (Epidemiology and Evolution of *Enterobacteriaceae* Infections in Humans and Domestic Animals) funded by the Wellcome Trust. The Scottish Agricultural College also acknowledges support from the Scottish Executive Environment and Rural Affairs Department (SEERAD).

We thank Doreen Bassett, Jude Evans, Hazel Knight, Judi Lee, and Alistair Smith for technical assistance, Loeske Kruuk for statistical assistance, and Ivan Morrison for calf immunity and development discussions.

REFERENCES

1. **Beutin, L., D. Geier, H. Steinrück, S. Zimmermann, and F. Scheutz.** 1993. Prevalence and some properties of verotoxin (Shiga-like toxin)-producing *Escherichia coli* in seven different species of healthy domestic animals. J. Clin. Microbiol. **31:**2483–2488.
2. **Blanco, J., M. Blanco, J. E. Blanco, A. Mora, M. P. Alonso, E. A. González, and M. I. Bernardez.** 2001. Epidemiology of verocytotoxigenic *Escherichia coli* (VTEC) in ruminants, p. 113–148. *In* G. Duffy, P. Garvey, and D. A. McDowell (ed.), Verocytotoxigenic *E. coli.* Food & Nutrition Press Inc., Trumbull, Conn.
3. **Boerlin, P., S. A. McEwen, F. Boerlin-Petzold, J. B. Wilson, R. P. Johnson, and C. L. Gyles.** 1999. Associations between virulence factors of Shiga toxin-producing *Escherichia coli* and disease in humans. J. Clin. Microbiol. **37:**497–503.
4. **Caprioli, A., A. E. Tozzi, G. Rizzoni, and H. Karch.** 1997. Non-O157 Shiga toxin-producing *Escherichia coli* infections in Europe. Emerg. Infect. Dis. **3:**578–579.
5. **Cobbold, R., and P. Desmarchelier.** 2000. A longitudinal study of Shiga-toxigenic *Escherichia coli* (STEC) prevalence in three Australian dairy herds. Vet. Microbiol. **71:**125–137.
6. **Cobbold, R., and P. Desmarchelier.** 2001. Characterisation and clonal relationships of Shiga-toxigenic *Escherichia coli* (STEC) isolated from Australian dairy cattle. Vet. Microbiol. **79:**323–335.
7. **Crawley, M. J.** 2002. Statistical computing: an introduction to data analysis using S-Plus. John Wiley & Sons, Chichester, United Kingdom.
8. **Dohoo, I., W. Martin, and H. Stryhn.** 2003. Veterinary epidemiological research. AVC Inc., Charolotte Town, Prince Edward Island, Canada.
9. **Dorn, C. R., D. H. Francis, E. J. Angrick, J. A. Willgohs, R. A. Wilson, J. E. Collins, B. H. Jenke, and S. J. Shawd.** 1993. Characteristics of verocytotoxin-producing *Escherichia coli* associated with intestinal colonisation and diarrhoea in calves. Vet. Microbiol. **36:**149–159.
10. **Frankel, G., A. D. Phillips, I. Rosenshine, G. Dougan, J. B. Kaper, and S. Knutton.** 1998. Enteropathogenic and enterohaemorrhagic *Escherichia coli*: more subversive elements. Mol. Microbiol. **30:**911–921.
11. **Friedrich, A. W., M. Bielaszewska, W. L. Zhang, M. Pulz, T. Kuczius, A. Ammon, and H. Karch.** 2002. *Escherichia coli* harbouring Shiga toxin 2 gene variants: frequency and association with clinical symptoms. J. Infect. Dis. **185:**74–84.
12. **Garber, L. P., S. J. Wells, D. D. Hancock, M. P. Doyle, J. Tuttle, J. A. Shere, and T. Zhao.** 1995. Risk-factors for faecal shedding of *Escherichia coli* O157:H7 in dairy calves. J. Am. Vet. Med. Assoc. **207:**46–49.
13. **Goddeeris, B.** 1998. Immunity in cattle, p. 439–484. *In* P.-P. Pastoret, P. Griebel, H. Barzin, and A. Govarts (ed.), Handbook of vertebrate immunology. Academic Press, London, United Kingdom.
14. **Gross, R. J., and B. Rowe.** 1985. Serotyping of *Escherichia coli*, p. 345–360. *In* M. Sussmann (ed.), The virulence of *Escherichia coli.* Cambridge University Press, Cambridge, United Kingdom.
15. **Jenkins, C., M. C. Pearce, A. W. Smith, H. I. Knight, D. J. Shaw, T. Cheasty, G. Foster, G. J. Gunn, G. Dougan, H. R. Smith, and G. Frankel.** 2003. Detection of *Escherichia coli* serogroups O26, O103, O111 and O145 from bovine faeces using immunomagnetic separation and PCR/DNA probe techniques. Lett. Appl. Microbiol. **37:**207–212.
16. **Kaper, J. B., S. Elliot, V. Sperandio, N. Perna, G. Mayhew, and F. Blattner.** 1998. Attaching and effacing intestinal histopathology and the locus of enterocyte effacement, p. 163–182. *In* J. B. Kaper and A. D. O'Brien (ed.), *Escherichia coli* and other Shiga toxin-producing *E. coli* strains. American Society for Microbiology, Washington, D.C.
17. **Karmali, M.** 1989. Infection by verocytotoxin-producing *Escherichia coli*. Clin. Microbiol. Rev. **2:**15–38.
18. **Kauffmann, F.** 1944. Zue serologie der Coli-Gruppe. Acta Pathol. Microbiol. Scand. **21:**20–45.
19. **Kobayashi, H., J. Shimada, M. Nakazawa, T. Morozumi, T. Pohjanvirta, S. Pelkonen, and K. Yamamoto.** 2001. Prevalence and characteristics of Shiga toxin-producing *Escherichia coli* from healthy cattle in Japan. Appl. Environ. Microbiol. **67:**484–489.
20. **Konowalchuk, J., J. I. Speirs, and S. Stavric.** 1977. Vero response to a cytotoxin of *Escherichia coli*. Infect. Immun. **18:**775–779.
21. **Lin, Z., S. Yamasaki, H. Kurazono, M. Ohmura, T. Karasawa, T. Inoue, S. Sakamoto, T. Suganami, T. Takeoka, Y. Taniguchi, and Y. Takeda.** 1993. Cloning and sequencing of two new verotoxin-2 variant genes of *Escherichia coli* isolated from cases of human and bovine diarrhoea. Microbiol. Immunol. **37:**451–459.
22. **Mainil, J.** 1999. Shiga verocytotoxins and Shiga verotoxigenic *Escherichia coli* in animals. Vet. Res. **30:**235–257.
23. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
24. **McMaster, C., E. A. Roch, G. A. Willshaw, A. Doherty, W. Kinnear, and T. Cheasty.** 2001. Verocytotoxin-producing *Escherichia coli* serotype 026:H11 outbreak in an Irish creche. Eur. J. Clin. Microbiol. Infect. Dis. **20:**430–432.
25. **Mohammad, A., J. S. M. Peiris, E. A. Wijewanta, S. Mahalingam, and G. Gunasekara.** 1985. Role of verocytotoxigenic *Escherichia coli* in cattle and buffalo calf diarrhoea. FEMS Microbiol. Lett. **26:**281–283.
26. **Naylor, S. W., J. C. Low, T. E. Besser, A. Mahajan, G. J. Gunn, M. C. Pearce, I. J. McKendrick, D. G. E. Smith, and D. L. Gally.** 2003. Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* 0157:H7 in the bovine host. Infect. Immun. **71:**1505–1512.

27. **O'Brien, A. D., J. W. Newland, S. F. Miller, R. K. Holmes, H. W. Smith, and S. B. Formal.** 1984. Shiga-like toxin-converting phages from *Escherichia coli* strains that cause hemorrhagic colitis or infantile diarrhoea. Science **226:** 694–696.
28. **Orden, J. A., J. A. Ruiz-Santa-Quiteria, D. Cid, S. García, R. Sanz, and R. de la Fuente.** 1998. Verotoxin-producing *Escherichia coli* (VTEC) and *eae*-positive non-VTEC in 1-30-days-old diarrhoeic dairy calves. Vet. Microbiol. **63:**239–248.
29. **Osek, J., P. Gallien, and D. Protz.** 2000. Characterisation of Shiga toxin-producing *Escherichia coli* strains isolated from calves in Poland. Comp. Immunol. Microbiol. Infect. Dis. **23:**267–276.
30. **Oswald, E., H. Schmidt, S. Morabito, H. Karch, O. Marchès, and A. Caprioli.** 2000. Typing of intimin genes in human and animal enterohemorrhagic and enteropathogenic *Escherichia coli*: characterization of a new intimin variant. Infect. Immun. **68:**64–71.
31. **Paton, A. W., R. M. Ratcliff, R. M. Doyle, J. Seymour-Murray, D. Davos, J. A. Lanser, and J. C. Paton.** 1996. Molecular microbiological investigation of an outbreak of hemolytic-uremic syndrome caused by dry fermented sausage contaminated with Shiga-like toxin-producing *Escherichia coli*. J. Clin. Microbiol. **34:**1622–1627.
32. **Pearce, M. C., C. Jenkins, L. Vali, A. W. Smith, H. I. Knight, T. Cheasty, H. R. Smith, G. J. Gunn, M. E. J. Woolhouse, S. G. B. Amyes, and G. Frankel.** 2004. Temporal shedding patterns and virulence factors of *Escherichia coli* serogroups O26, O103, O111, O145, and O157 in a cohort of beef calves and their dams. Appl. Environ. Microbiol. **70:**1708–1716.
33. **Piérard, D., G. Muyldermans, L. Moriau, D. Stevens, and S. Lauwers.** 1998. Identification of new verocytotoxin type 2 variant B-subunit genes in human and animal *Escherichia coli* isolates. J. Clin. Microbiol. **36:**3317–3322.
34. **Pinheiro, J. C., and D. M. Bates.** 2000. Mixed-effects models in S and S-plus. Springer-Verlag, New York, N.Y.
35. **Roy, J. H. B.** 1980. The calf. Butterworth, London, United Kingdom.
36. **Sandhu, K. S., and C. L. Gyles.** 2002. Pathogenic Shiga toxin-producing *Escherichia coli* in the intestine of calves. Can. J. Vet. Res. **66:**65–72.
37. **Scheutz, F., L. Beutin, D. Piérard, and H. Smith.** 2001. Nomenclature of verocytotoxins, p. 447–452. *In* G. Duffy, P. Garvey, and D. A. McDowell (ed.), Verocytotoxigenic *E. coli.* Food & Nutrition Press Inc., Trumbull, Conn.
38. **Schmidt, H., L. Beutin, and H. Karch.** 1995. Molecular analysis of the plasmid-encoded hemolysin of *Escherichia coli* O157:H7 strain EDL-933. Infect. Immun. **63:**1055–1061.
39. **Scotland, S. M., H. R. Smith, G. A. Willshaw, and B. Rowe.** 1983. Vero cyto-toxin production in strain of *Escherichia coli* is determined by genes carried on bacteriophage. Lancet **ii:**216.
40. **Sherwood, D., D. R. Snodgrass, and A. D. O'Brien.** 1985. Shiga-like toxin production from *Escherichia coli* associated with calf diarrhoea. Vet. Rec. **116:**217–218.
41. **Snodgrass, D. R., H. R. Terzolo, D. Sherwood, I. Campbell, J. D. Menzies, and B. A. Synge.** 1986. Aetiology of diarrhoea in young calves. Vet. Rec. **119:**31–34.
42. **Synge, B. A., M. E. Chase-Topping, G. F. Hopkins, I. J. McKendrick, F. Thomson-Carter, D. Gray, S. M. Rusbridge, F. I. Munro, G. Foster, and G. J. Gunn.** 2003. Factors influencing the shedding of verocytotoxin-producing *Escherichia coli* O157 by beef suckler cows. Epidemiol. Infect. **130:**301–312.
43. **Thomas, A., H. R. Smith, G. A. Willshaw, and B. Rowe.** 1991. Non-radioactively labeled polynucleotide and oligonucleotide DNA probes, for selectively detecting *Escherichia coli* strains producing verocytotoxins VT1, VT2 and VT2 variant. Mol. Cell. Probes **5:**129–135.
44. **Tozzi, A. E., S. Goretti, and A. Caprioli.** 2001. Epidemiology of human infections by *Escherichia coli* and other verocytotoxin-producing *E. coli*, p. 161–180. *In* G. Duffy, P. Garvey, and D. A. McDowell (ed.), Verocytotoxigenic *E. coli.* Food & Nutrition Press Inc., Trumbull, Conn.
45. **Wells, J. G., L. D. Shipman, K. D. Greene, E. G. Sowers, J. H. Green, D. N. Cameron, F. P. Downes, M. L. Martin, P. M. Griffin, S. M. Ostroff, M. E. Potter, R. V. Tauxe, and I. K. Wachsmuth.** 1991. Isolation of *Escherichia coli* serotype O157:H7 and other Shiga-like-toxin-producing *Escherichia coli* from dairy cattle. J. Clin. Microbiol. **29:**985–989.
46. **Wieler, L. H., R. Bauerfeind, and G. Baljer.** 1992. Characterisation of Shiga-like toxin producing *Escherichia coli* (SLTEC) isolated from calves with and without diarrhoea. Int. J. Med. Microbiol. Virol. Parasitol. Infect. Dis. **276:** 243–253.
47. **Willshaw, G. A., T. Cheasty, H. R. Smith, S. J. O'Brien, and G. K. Adak.** 2001. Verocytotoxin-producing *Escherichia coli* (VTEC) O157 and other VTEC from human infections in England and Wales: 1995–1998. J. Med. Microbiol. **50:**135–142.
48. **Willshaw, G. A., S. M. Scotland, H. R. Smith, and B. Rowe.** 1992. Properties of verocytotoxin-producing *Escherichia coli* of human-origin of O-serogroups other than O157. J. Infect. Dis. **166:**797–802.
49. **Willshaw, G. A., H. R. Smith, D. Roberts, J. Thirlwell, T. Cheasty, and B. Rowe.** 1993. Examination of raw beef products for the presence of verocytotoxin-producing *Escherichia coli*, particularly those of serogroup O157. J. Appl. Bacteriol. **75:**420–426.
50. **Willshaw, G. A., H. R. Smith, S. M. Scotland, A. M. Field, and B. Rowe.** 1987. Heterogeneity of *Escherichia coli* phages encoding verocytotoxins—comparison of cloned sequences determining VT1 and VT2 and development of specific gene probes. J. Gen. Microbiol. **133:**1309–1317.
51. **Wilson, J. B., S. A. McEwen, R. C. Clarke, K. E. Leslie, R. A. Wilson, D. Walter-Toews, and C. L. Gyles.** 1992. Distribution and characteristics of verocytotoxigenic *Escherichia coli* isolated from Ontario dairy cattle. Epidemiol. Infect. **108:**423–439.
52. **World Health Organization.** 1998. Zoonotic non-O157 Shiga-toxin producing *Escherichia coli* (STEC). W.H.O./EMC/ZDI/98.8. World Health Organization, Geneva Switzerland.
53. **Zhang, W. L., M. Bielaszewska, T. Kuczius, and H. Karch.** 2002. Identification, characterization, and distribution of a Shiga toxin 1 gene variant (stx_{1c}) in *Escherichia coli* strains isolated from humans. J. Clin. Microbiol. **40:**1441–1446.
54. **Zhang, W. L., B. Köhler, E. Oswald, L. Beutin, H. Karch, S. Morabito, A. Caprioli, S. Suerbaum, and H. Schmidt.** 2002. Genetic diversity of intimin genes of attaching and effacing *Escherichia coli* strains. J. Clin. Microbiol. **40:**4486–4492.